

# Tumor Necrosis Factor- $\alpha$ Receptor II Polymorphism in Patients from Southern Europe with Mild–Moderate and Severe Rheumatoid Arthritis

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**ABSTRACT. Objective.** To define the frequency of the exon 6 tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) receptor II (TNFRII) gene polymorphism in severe and mild–moderate rheumatoid arthritis (RA) and its possible influence on anti-TNF- $\alpha$  treatment responsiveness.

**Methods.** Two cohorts of patients with RA, the first ( $n = 97$ ) defined as methotrexate responders (MTX-R) with mild–moderate synovitis, and the second ( $n = 78$ ) defined as nonresponders to combination therapy and receiving anti-TNF- $\alpha$  treatment because of their severe and aggressive disease (TNF-T), were studied retrospectively and compared to age, sex, and ethnically matched controls ( $n = 84$ ). In the prospective study, 66 patients with severe RA were followed over the first 6 months of anti-TNF- $\alpha$  therapy and their response was examined according to genotype.

**Results.** We observed a trend towards an increased frequency of the GG genotype in patients with severe RA (6.4%) in comparison with patients with mild–moderate disease (3.1%) and controls (1.2%). When looking at the response to anti-TNF- $\alpha$  therapy, we observed that after 12 weeks of treatment, 37.8% of the TT versus 10.7% of the TG/GG patients passed from high to medium–low disease activity ( $p = 0.03$ ).

**Conclusion.** In our cohorts of patients selected by response to the conventional therapy and by disease severity, our preliminary study results showed a trend towards a higher prevalence of the GG genotype for the exon 6 TNFRII polymorphism in the less responsive patients with more aggressive disease. We also found a lower degree of response to anti-TNF- $\alpha$  treatments in patients carrying the G allele. (J Rheumatol 2002;29:1847–50)

## Key Indexing Terms:

RHEUMATOID ARTHRITIS

TUMOR NECROSIS FACTOR- $\alpha$

TUMOR NECROSIS FACTOR- $\alpha$  RECEPTOR II POLYMORPHISM

Rheumatoid arthritis (RA), like many other autoimmune diseases, i.e., diabetes, is characterized by an important genetic component. The heritability from twin and familial studies has been set at 60% of all predisposing factors<sup>1–6</sup>. Of this 60%, about 40% are related to HLA genes<sup>5</sup>.

Recent studies by the ECRAF (European Consortium of Rheumatoid Arthritis Families)<sup>7</sup> have shown that the strongest association resides certainly in the HLA genes, but there are 19 other possible markers in 14 different chromosomal regions. Among these, a crucial role seems to be played by genes in chromosome 3, possibly explaining 16% of the genetic heritage of the disease. On the other hand, the

NARAC (North American Rheumatoid Arthritis Consortium)<sup>8</sup> has revealed significant associations with loci localized in chromosomes 1, 4, 12, 16, and 17. Both ECRAF and NARAC studies found some important loci in chromosome 1, and among the genes that might be involved in the disease we have investigated the TNF receptor II (TNFRII) gene<sup>9,10</sup>. A recent report from Barton, *et al*<sup>11</sup> suggested an association between the single nucleotide polymorphism (SNP) at position 196 in exon 6 of the TNFRII gene and familial RA in particular.

## MATERIALS AND METHODS

**Retrospective study.** One hundred seventy-five patients with RA of Italian Caucasian origin and 84 sex, age, and ethnically matched healthy blood donor controls entered the study. RA was diagnosed on the basis of the American College of Rheumatology 1987 criteria<sup>12</sup>. Patients with RA were split into 2 subgroups as described<sup>13</sup>. Group A (97 patients) included patients with RA in stable partial remission for at least 6 months after weekly methotrexate (MTX) treatment at a dose of 15 mg/week (range 10–25 mg/week). Stable partial remission was defined as less than 3 swollen joints and morning stiffness < 20 min. This group was labeled MTX responders (MTX-R). Group B (78 patients) included patients with RA with active disease despite 6 months of combination therapy [MTX + sulfasalazine (SSZ) + hydroxychloroquine (HCQ)<sup>13</sup> or MTX + cyclosporin

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A (CSA)<sup>14</sup>, with optional low doses of prednisone, 5 mg/day]. Active disease was defined as persistently more than 6 swollen joints and morning stiffness  $\geq 60$  min. This group would receive anti-TNF- $\alpha$  therapy and was labeled as anti-TNF treated (TNF-T). Demographic and clinical features are reported in Table 1.

**Prospective study.** Sixty-six consecutive patients with severe RA (in the TNF-T subgroup) were given anti-TNF- $\alpha$  therapy (etanercept 25 mg twice weekly or infliximab according to the ATTRACT schedule<sup>15</sup>) and were followed over 6 months. We calculated the Disease Activity Score (DAS) value at baseline and at 4, 12, and 24 weeks of treatment.

**DNA isolation and genotyping.** Genomic DNA was extracted from 7 ml of anticoagulated blood by the salting out method. The -238 and +489 TNF- $\alpha$  gene polymorphisms were analyzed as reported<sup>16</sup>, while the T/G polymorphism in exon 6 of the TNFR2 gene was analyzed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method indicated by Al-Ansari, *et al*<sup>17</sup>. As illustrated in Figure 1, when the T allele but not the G is present, the Nla III restriction enzyme cleaves the 242 base pair PCR product, generating 2 smaller fragments of 133 and 109 base pairs.

**Table 1.** Demographic and clinical characteristics of nonresponder (TNF-T) and responder (MTX-R) patients with RA.

Variables	TNF-T, n = 78	MTX-R, n = 97
Age, yrs*	54.5 (25–79)	61 (17–81)
Sex, % female	93.6	85
Age at onset, yrs*	41 (14–70)	49 (13–76)
Disease duration, yrs*	11 (1–40)	8 (1–51)
Swollen joint count*	12 (6–55)	2 (0–3)
Tender joint count*	18 (6–68)	3 (0–51)
RF positivity, %	67.1	54
CRP, mg/l*	27.5 (10–120)	4.8 (0.3–50)
Steinbrocker functional class, %		
I	23.7	69.1
II	39.5	28.4
III	22.3	2.5
IV	14.5	
-238 TNF- $\alpha$ polymorphism genotype frequencies**, %		
GG	100	92.8
AG		7.2
+489 TNF- $\alpha$ polymorphism genotype frequencies**, %		
GG	66.6	68.2
AG	29.5	30.3
AA	3.9	1.5

\* Median (min–max); RF: rheumatoid factor (positive if  $> 40$  IU/ml); CRP: C-reactive protein (normal if  $< 5$  mg/l); \*\* as reported<sup>16</sup>.

**Statistics.** Statistical analyses (odds ratios, 95% confidence intervals) were performed using Prism software (Graph-Pad, San Diego, CA, USA). We calculated the p value by Yates' continuity corrected chi-square test (p significant if  $< 0.05$ ).

## RESULTS

**TNFR2 polymorphism in patients and controls.** As illustrated in Table 2, the frequency of the GG genotype was higher in patients with severe RA (6.4%) than in the mild-moderate group (3.1%) and in controls (1.2%), but the differences were not significant (OR TNF-T vs control 5.7, 95% CI 0.65–50,  $p = 0.17$ ). Even if the G allele frequency was slightly increased in TNF-T patients (24.3%) with respect to MTX-R (19.6%) and controls (16.7%), the frequency of the heterozygotic subjects was exactly the same (33%).

**TNFR2 polymorphism and anti-TNF agent responsiveness.** Sixty-six consecutive patients with severe RA among the cohort of severe RA (38 TT, 22 TG, and 6 GG individuals) were followed over the first 6 months of anti-TNF therapy (etanercept: 19 cases, of whom 10 were TT; and infliximab: 47 cases, of whom 28 were TT) and their clinical responses were evaluated calculating the DAS value at baseline and at 4, 12, and 24 weeks of therapy. The DAS value defines the level of disease activity as high (DAS  $> 3.7$ ), medium (2.4  $<$  DAS  $< 3.7$ ) and low (DAS  $< 2.4$ )<sup>18</sup>. All except one of the patients (a TT patient with DAS = 3.07) had started the anti-TNF therapy with high disease activity. In Figures 2A and 2B, at each considered time point, we have illustrated the percentage of patients in each DAS range according to the TNFR2 genotype: TT or TG/GG. The TT subjects always had a higher level of responsiveness (medium-low DAS) than TG/GG subjects. If we calculate the OR between the TT and TG/GG subjects in the medium-low DAS ranges versus the high range, we find that the greatest difference appears at 12 weeks (37.8 vs 10.7%; OR 5.1, 95% CI 1.3–19.96,  $p = 0.03$ ). Thus, the presence of at least one G allele seems to predispose towards a less responsive phenotype during anti-TNF therapy.

The TNFR2 G allele associates with other unfavorable genetic assets in patients with RA. We previously analyzed 2 important TNF- $\alpha$  gene polymorphisms in RA<sup>16</sup>, and found an absolute segregation of the GG genotype for the -238

**Table 2.** Exon 6 TNFR2 polymorphism genotyping: genotype and allele frequencies in controls and in the 2 subgroups of patients with RA. Odds ratios, confidence intervals, and p values (Yates' continuity corrected chi-square test, 2 sided) regarding the GG genotype frequency in RA subgroups versus controls are reported.

	TT, % (n)	TG, % (n)	GG, % (n)	G Allele, %	T Allele, %	n	OR (95% CI)	p
Controls	67.8 (57)	31.0 (26)	1.2 (1)	16.7	83.3	84		
RA total	61.1 (107)	34.3 (60)	4.6 (8)	21.7	78.3	175	4.0 (0.49–32)	NS
MTX-R	63.9 (62)	33 (32)	3.1 (3)	19.6	80.4	97	2.7 (0.27–26)	NS
TNF-T	57.7 (45)	35.9 (28)	6.4 (5)	24.3	75.7	78	5.7 (0.65–50)	NS

n: number of subjects.

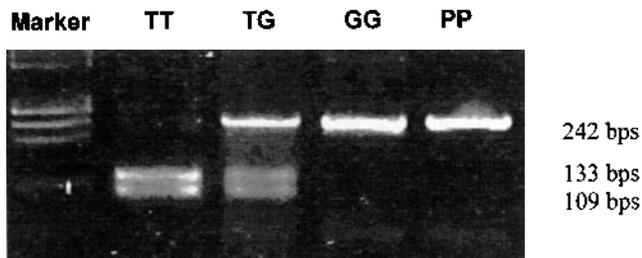


Figure 1. Exon 6 TNFR2 gene polymorphism genotyping through Nla III digestion of the PCR product (PP). Marker: phiX Hae III.

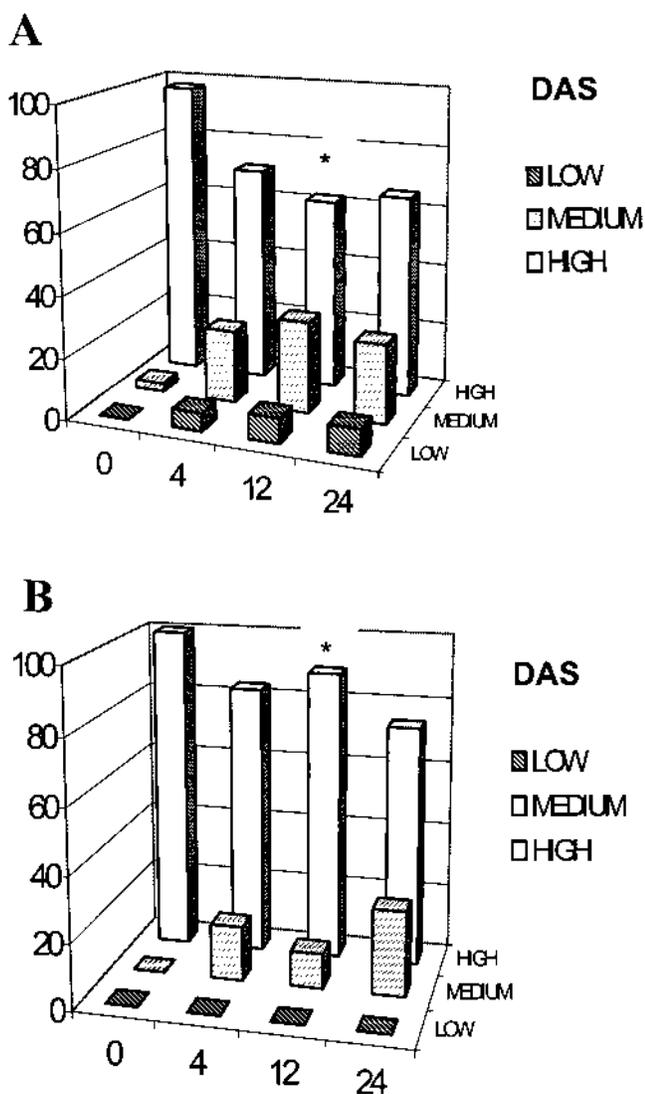


Figure 2. Disease activity variation under anti-TNF therapy in 38 TT (A) and 28 TG/GG (B) patients with RA. x axis: weeks of treatment (zero means baseline status). y axis: percentage of patients with high, medium, and low disease activity (calculated by the DAS value). \* $p = 0.03$ .

TNF- $\alpha$  gene polymorphism in patients with severe RA, while the MTX-R patients were indistinguishable from controls (about 92% GG). Moreover, we observed a sort of protective effect of the +489 TNF- $\alpha$  polymorphism A allele, even if its decreased presence in severe RA in particular was not statistically significant. The genotypic analysis for these polymorphisms in our RA patients is reported in Table 2. Assuming that the +489 GG patients are less protected from a worse clinical course and radiographic damage, we analyzed only these patients in each subgroup in order to disclose a possible cooperative effect between this TNF polymorphism and the TNFR2 G allele. We observed no difference in the percentage of TNFR2 exon 6 G allele-carrying patients between the 2 +489 GG RA subgroups (38%), but they were different from the controls (25%). Thus among the +489 GG subjects the presence of the G TNFR2 allele seems to increase the predisposition to RA, but not to a more aggressive disease course.

## DISCUSSION

Our study shows a trend towards an association between the TNFR2 exon 6 polymorphism GG genotype and severe RA. The frequencies of the genotypes in our southern European population differ slightly from those reported by Barton, *et al* (Northern Europe)<sup>11</sup>, as reported for the HLA genes<sup>5</sup>. We also noted an increased frequency in the GG homozygosity in RA in general. While Barton, *et al* had designed a case control study in which RA patients were stratified according to the presence of a family history for the disease and showed that the increased frequency resides in the familial RA, we divided *a posteriori* our patients into 2 cohorts, one characterized by an excellent response to MTX treatment and by a mild-moderate disease activity (defined as MTX responders, MTX-R), the other by high disease activity and lack of response to the commonly used combination therapies. Because of this failure to respond, all these patients with severe RA were to receive anti-TNF- $\alpha$  therapy and were defined as TNF treated (TNF-T). Looking at the TNFR2 exon 6 polymorphism analysis (Table 2), we note a progressive increment in the GG genotype frequency comparing controls (1.2%), MTX-R (3.1%), and TNF-T patients (6.4%). Even though the OR between TNF-T and controls is very high (5.7), the difference did not reach statistical significance, probably because of the low frequency of the GG genotype and the insufficient number of subjects analyzed for this purpose. We calculated that we would have to extend the genotypic analysis to an additional 50 patients with severe RA (severe RA represents 33% of our total RA population) and to 50 additional controls to reach the statistical significance with Yates' correction. If we couple data from Barton, *et al* that suggest an influence of this genotype on the clinical course of the disease with our data that have revealed a possible link between the TNFR2 GG genotype and a poor responsive phenotype, we

might hypothesize an early identification of a subset with worse outcome after few months of MTX treatment.

The functional relevance of the T/G SNP in exon 6 of the TNFR2 gene is still unknown. The working hypothesis is that the methionine to arginine substitution could affect membrane receptor shedding and/or ligand binding<sup>9,10</sup>. Moreover it is still an open question whether the heterozygotic or the homozygotic asset is necessary to determine a different phenotype. In this regard, our preliminary data on the response to anti-TNF- $\alpha$  therapy seems to suggest a possible influence of the G allele, also in the heterozygotic asset. Indeed, following for the first 6 months of therapy 23 TG plus 5 GG and 38 TT patients with RA, we found a greater percentage of responsive subjects in the TT subgroup after 12 weeks of treatment (37.8 vs 10.7%;  $p = 0.03$ ). This might mean that one G allele is enough to determine an unresponsive phenotype, but we certainly need a larger study to confirm this observation.

We recently analyzed 2 TNF- $\alpha$  gene polymorphisms, finding that patients with severe RA were all GG for the -238 polymorphism and presented a reduced frequency of the protective A allele of the +489 polymorphism<sup>16</sup>. Considering all the -238 GG subjects (given the very small number of -238 AG cases) we tried to analyze the possible effect of the combined absence of the protective +489 A allele and the presence of at least one G TNFR2 allele. The percentage of individuals not carrying the +489 A allele that present at least one G TNFR2 allele was higher in RA in general (38%) compared to controls (25%), but did not differ between the 2 cohorts of patients with RA. Thus the association between these 2 unfavorable genetic assets might predispose to RA development, but should not influence the disease course, even if individually these allelic variants seem to characterize patients with more severe and unresponsive disease.

We show that in a Mediterranean cohort of patients with RA, the TNFR2 exon 6 polymorphism GG genotype tends to characterize a worse disease course and preliminary evidence suggests that the presence of the G allele could negatively affect the response to anti-TNF therapeutic strategies.

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